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EARLY POST-METAMORPHIC GROWTH, BUDDING AND SPICULE FORMATION IN THE COMPOUND ASCIDIAN *CYSTODYTES LOBATUS*

GRETCHEN LAMBERT

Department of Biological Science, California State University, Fullerton, California 92634 and Hopkins Marine Station of Stanford University, Pacific Grove, California 93950

Compound ascidian colonies are comprised of many individuals wholly embedded in a common tunic. Budding is accomplished by active epidermal synthesis and constriction, with the regenerating buds moving through this common tunic to form a new system (see Berrill, 1951, 1961 for reviews of early papers; Sebastian, 1957; Levine, 1960; Freeman, 1971; Nakauchi, 1966a, b, c, 1970, 1977; Nakauchi and Kawamura, 1974a, b, 1978). *Cystodytes,* unlike other genera in the suborder Aplousobranchia, family Polycitoridae, is distinguished by large numbers of calcareous spicules surrounding the abdomen of each zooid, forming a spicular sac separating each individual from neighboring zooids (Ritter, 1900; Van Name, 1945). During budding this spicular sac must be reorganized and reformed around the abdomen of each bud, but the way in which this is accomplished is completely unknown. The common tunic overlying the spicular layer contains numerous large, closely packed, acid-filled bladder cells (Abbott and Newberry, 1980); any rupture of these cells results in immediate dissolution of nearby spicules and evolution of CO_2. The spicules might be dissolved and reformed during budding or just reallocated in some way among the buds; there appear to be no life history studies on any species of the genus *Cystodytes,* even though the genus is common and widespread (Van Name, 1945; Millar, 1975). Therefore, the present study is an examination of the general ecology and method of budding in *Cystodytes lobatus* (Ritter, 1900), a species that occurs abundantly in the low intertidal and subtidal zones along the central California coast. The time course of tadpole release, spicule formation in oozooids (the individuals developing from newly-settled tadpoles before the first budding), and the method of budding with each zooid isolated in its own spicular sac are discussed here. Included is the discovery that the spicules are contained within a discrete extra-cellular membrane. This membrane has been named the tunic spicular lamina and is concluded to form an organic matrix for spicule formation.

Materials and Methods

This work was carried out at the Hopkins Marine Station, Pacific Grove, California, between February and August, 1978. Animals were collected from rocks in front of the marine station and at Pt. Pinos, about two miles away. Three color variants exist: white, pink and orange. Since the taxonomy of these variants has not been studied, and since Ritter (1900) based his original description of the species on the white form, only white colonies were used throughout this study.

Colonies were maintained in unfiltered running sea-water aquaria in the laboratory; between February and August the temperature varied only from 13° to 16° C. Colonies usually lived at least a month in the laboratory, and if they reattached to the bottom of the aquarium they survived even longer.

Colonies with tadpoles were easily recognized by the presence of the bright pink yolky embryos. Since it is known that some colonial ascidians release their tadpoles in response to light (*e.g.*, Watanabe and Lambert, 1973), the colonies were kept in the dark at night. Tadpole collection was accomplished by removing the colonies in the morning to a clear plastic aquarium supplied with running sea water. Drainage was through a hose penetrating the aquarium at one end, with water flow adjusted so that the water level was at the hose exit. In this way, as tadpoles were released from the colonies and swam upwards they were skimmed off and carried through the hose to a tadpole collector, consisting of a short piece of polyvinyl chloride pipe with 300-μm Nytex glued to the bottom of it and resting in a small dish. Tadpoles were released throughout the day. Periodically they were removed from the collector to a petri dish with a glass slide in the bottom of it centered over a piece of black plastic under the dish to induce settling of the tadpoles in the darkest area. These slides were then placed in a plexiglass slide holder and submerged in a large cement tank in the laboratory filled with running unfiltered sea water where they were maintained for several months. Slides were removed from the holder and placed in a sea water-filled petri dish for examination of the living zooids, using an American Optical dissecting microscope with phototube. All photographs were taken with an Olympus OM-2 35-mm SLR camera with microscope adapter.

A Beckman Expandomatic IV pH meter equipped with a rapid-response MI-410 combination pH probe (Microelectrodes, Inc., Londonderry, N. H.) was used for all pH determinations. Bladder cell pH was determined in two ways. Adult colonies were washed several times in distilled water, blotted dry with Kimwipes, then either the pH electrode was carefully inserted about 2 to 4 mm into the superficial layer of the tunic or bladder cells in the upper tunic layer were broken by agitation with a fine probe and then the pH electrode was immersed directly into the resulting pool of (mostly) bladder cell fluid. Results were the same for both methods. Bladder cell contents were analyzed for the presence of chloride and sulfate ions by probing them directly with hand-made finely drawn out glass micropipettes under a dissecting microscope. The fluid collected was expelled into a watch glass, and a drop of either silver nitrate or barium chloride was added. ($AgNO_3$ forms a precipitate of $AgCl_2$ in the presence of chloride ions, $BaCl_2$ forms a precipitate of $BaSO_4$ in the presence of sulfate ions.)

Because alcohol dehydration and even fixation in buffered formalin did not prevent rupture of bladder cells and dissolution of spicules, razor blade sections of living colonies were made and stained supravitally with PAS, aldehyde fuchsin, alcian blue pH 2.5, alcian blue pH 1.0, sudan black B, 0.1% toluidine blue, or neutral red, using the methods in Pearse (1968), in order to analyze the tunic for acid mucopolysaccharides and other structural materials.

A few 4-wk-old oozooids settled on slides were relaxed in 100 ml sea water to which had been added 1 drop of menthol-saturated 95% ethanol, according to the method of Abdel-Malek (1951). When total relaxation was achieved in 3 to

6 hr. 10% formalin was added drop by drop while stirring, until the animals were dead. The zooids were transferred to 10% formalin for 24 hr, then removed from their slides, washed, dehydrated in alcohols and embedded in the Polysciences JB4 plastic embedding medium. After 24 hr the plastic blocks were trimmed and sectioned at 1 to 2 μm. Sections were removed one at a time, placed in a drop of 1% ammonia on a slide to flatten out, then flame-dried and stained with methylene blue or 0.1% toluidine blue.

Results

General biology

Cystodytes lobatus grows in large mats, up to a half meter across or more; whether each mat is one colony or many that have fused or abutted was not determined. Colonies by the Hopkins Marine Station averaged 5 mm in thickness. However, material collected elsewhere and in D. P. Abbott's private collection may be up to 1 cm or more in thickness (Abbott and Newberry, 1980).

Colonies always occurred in the low intertidal or subtidal; a −0.6 ft tide or lower was necessary to collect intertidally. The best intertidal sites were under overhanging rocks and to a lesser extent on the north side of vertical rocks, away from direct sunlight and (possibly) from competition with plants.

Large colonies were commonly observed to have overgrown many barnacles and polychaete worm tubes, resulting in a mat with many superficial ridges and knobs. Cross sections indicated colonies to be of variable thickness; some ridges covered overgrown barnacles while others were present without any underlying irregularity of the substratum. Other compound ascidians, common in the same area as *Cystodytes*, were *Aplidium californicum* and *Archidistoma psammion;* colonies of both these species also attained large size, and a dynamic situation appeared to be present of overgrowth of one ascidian by another—either overgrowth of *Cystodytes* by *Aplidium* or *Archidistoma* or vice versa.

Very few predators were observed feeding on *C. lobatus;* those animals found on or near colonies in the field were the starfish *Patiria miniata* and the gastropods *Calliostoma ligatum, Tegula funebralis, Megathura crenulata,* and *Lamellaria diegoensis.* However, these observations were made intertidally at low tide when *Cystodytes* was out of water; high tide observations might be different. When *Cystodytes* is out of water it forms a slimy coating of an (apparently) mucus-like material that can be seen hanging in long strands and dripping off the colonies. This material is not present in submerged colonies until the surface is rubbed. The significance of this is as yet unknown.

Laboratory feeding observations in the above-mentioned animals found on or near colonies showed that all but *Tegula funebralis* would eat *Cystodytes* in the laboratory. Stomach contents of subtidal *Calliostoma ligatum* and *Megathura crenulata* collected on or near *Cystodytes* yielded chunks of colony with the spicules mostly intact (Sellers, 1977 and personal communication). *Lamellaria diegoensis* was maintained in the laboratory from March 10 until August 31 on a diet of nothing but *Cystodytes lobatus*; during this period its weight increased from 2.2 grams (May 3) to 6.0 grams (August 21). A study of feeding of

FIGURE 1. Two *Cystodytes lobatus* systems comprised of 5 and 3 zooids. Scale bar 1 mm.

Lamellaria on *Cystodytes,* including a calorific analysis of *Cystodytes* colonies utilizing a semimicro bomb calorimeter, will be published separately (Laubert, 1979).

Other animals associated with *Cystodytes* as well as other colonial ascidians are the clam *Mytilimeria nuttalli* and the amphipod *Polycheria osborni,* both of which live embedded in the ascidian test (Abbott and Newberry, 1980). Very little is known of the relationship of these species to their ascidian hosts (Skogsberg and Vansell, 1928; Yonge, 1952).

Colony organization and tadpole release

The zooids of *Cystodytes* (Fig. 1) are arranged in systems (Van Name, 1945) with a mean of 4 or 5 zooids per system (Fig. 2). The atrial siphons open separately at the surface (Fig. 1). The abdomens are surrounded by a layer of overlapping calcareous spicules (Figs. 3, 4) and the test matrix is filled with bladder cells (Van Name, 1945) tightly packed and ranging in size roughly from 35 to 80 μm. These cells are filled with sulfuric and possibly hydrochloric acids; tests were positive for the presence of both sulfate (Abbott and Newberry, 1980)

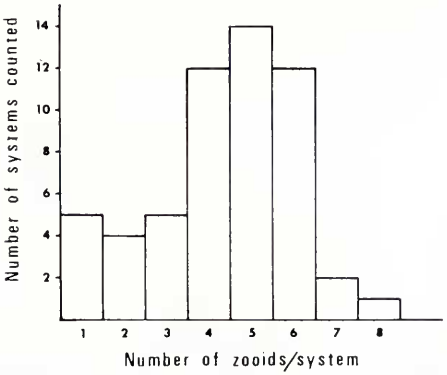

FIGURE 2. Number of zooids per system. n = 55 systems, $\bar{x}$ = 4.36 zooids/system, s.d. = 1.69.

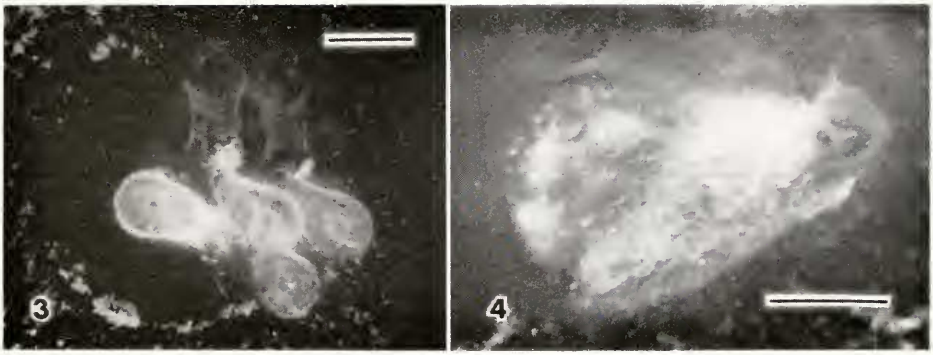

FIGURE 3. Underside of a *C. lobatus* system. Scale bar 1 mm.
FIGURE 4. *C. lobatus* oozooid 32 days old. Scale bar 0.5 mm.

and chloride ions. The pH of bladder cell contents was determined to be 1.3.

Embryos develop within the atrial chamber of the adult zooids (Van Name, 1945). Because of their bright pink color they could be observed easily and were recorded in nearly all colonies collected between March 7 and August 18.

Tadpoles were collected in the tadpole collector and settled on glass in order to observe the growth of zooids in the laboratory, the time course of spicule and bladder cell formation, and the method of budding. The time course of tadpole release (Fig. 5) reveals that more tadpoles were released after 3 to 4 hr in the light following overnight dark adaption under black plastic than at any other time, and tadpoles were released more or less continuously all day. Colonies left in an uncovered aquarium overnight in order to observe their reaction to natural dawn also released larvae sporadically throughout the day.

Cystodytes tadpoles are large, with a mean body length of 1.27 mm ($n = 25$, s.d. = 0.113), tail length of 2.59 mm ($n = 25$, s.d. = 0.141), and tail width of

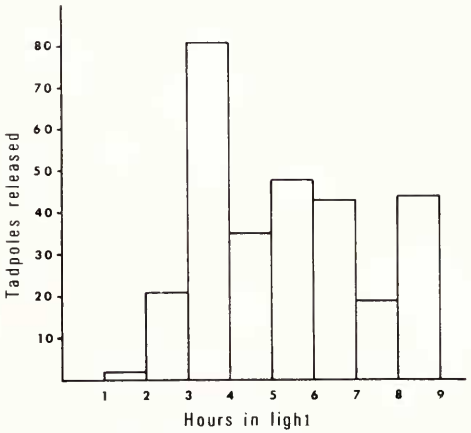

FIGURE 5. Tadpole release as related to duration of light period after darkness. Numbers are totals for 10 days of observations.

0.76 mm (n = 25, s.d. = 0.049). The heart beats somewhat erratically but does reverse, as in the tadpoles of *Distaplia* and *Diplosoma* (Cloney, personal communication) and *Pycnoclavella stanleyi* (Trason, 1963). Bladder cells are already densely packed in the test matrix of the tadpole. The pH of six individuals homogenized in a few drops of distilled water was 2.85. A few tadpoles metamorphosed within 15 min of being released, though most had a free larval life of 1 to 3 hr. Settlement was greatly enhanced by using slides that had been soaked in sea water for several days.

Post-metamorphic growth and budding

Young oozooids begin to feed 3 to 4 days after settlement (as determined by the presence of food pellets in the gut) and by one week of age the gut has differentiated into five well-defined regions similar to those in many aplousobranchs: esophagus, stomach, post-stomach, mid-intestine, and intestine or rectum. The stomach and intestine are orange-brown; the rest of the zooid is colorless or nearly so. By 24 hr or so after settlement, oozooids have four rows of stigmata, the same as the adult blastozooids. This is similar to the closely related polycitorid *Archidistoma ritteri* (Levine, 1960) in which both oozooids and blastozooids have three rows. In the polyclinid *Amaroucium multiplicatum* (Nakauchi, 1966a), in contrast, the oozooids have four rows but after budding the blastozooids have six or seven rows of stigmata. In *C. lobatus* there are usually four stigmata per side in the anteriormost row at metamorphosis; at budding this row has 17 or 18 stigmata per side. With successive buddings the blastozooids orient vertically with the abdomen directly beneath the thorax rather than curved around it is in the oozooid, but otherwise the oozooids and blastozooids appear to be the same morphologically. Whether oozooids ever from gonads, however, was not determined.

There is great variation among oozoids in the quantity of spicules produced, even those arising from tadpoles from a single colony reared on the same slide (compare Fig. 4 with Figs. 6 and 8). A number of tadpoles were collected on May 25 and allowed to settle on three slides which were maintained in the

TABLE I.

Relationship between spicule density and time to budding, number of abdominal buds and time until buds begin feeding in C. lobatus.

	n	$\bar{x}$	s.d.
Days from settlement to budding for zooids with few spicules	32	29.72	3.0
Days to budding for zooids with many spicules	18	37.33	6.28
Number of buds, few-spiculed zooids	32	3.59	0.61
Number of buds, many-spiculed zooids	18	2.89	0.47
Days from budding until buds begin feeding in few-spiculed zooids	24	5.58	0.50
Days from budding until buds begin feeding, many-spiculed zooids	15	5.67	0.62

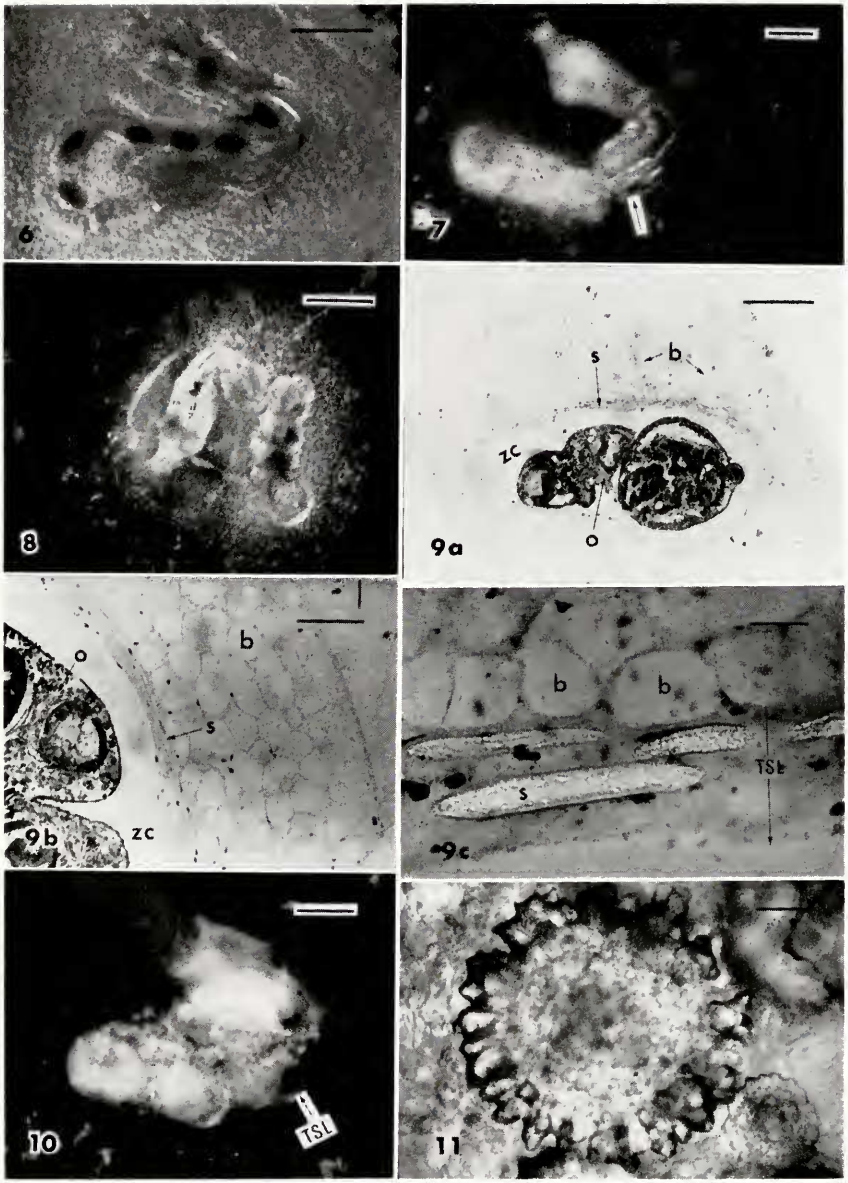

FIGURE 6. Twenty-five-day-old oozooid. Arrow indicates position of new stomach. Scale bar 0.5 mm.

FIGURE 7. Thirty-one-day-old oozooid. Arrow indicates new stomach at posterior end of mother bud. Scale bar 0.5 mm.

FIGURE 8. Thirty-three-day-old oozooid; budding is nearly complete. Scale bar 1 mm.

FIGURE 9. Four-week-old oozooids, 1 μm sections. (a) entire zooid; scale bar 250 μm. (b) Enlargement of tunic region; scale bar 100 μm. (c) TSL region of tunic; scale bar 20 μm. b = bladder cell, o = oozooid, s = spicule, TSL = tunic spicular lamina, zc = zooid cavity.

FIGURE 10. Thirty-six-day-old oozooid forming 3 abdominal buds; scale bar 0.5 mm. B = bud, TSL = disrupted tunic spicular lamina.

FIGURE 11. Spicule from an adult *C. lobatus* colony. Scale bar 50 μm.

laboratory until the end of August. Table I compares oozooids that developed few spicules with those that developed many. Zooids with many spicules took longer to bud (37 days as compared with 30 days) and produced fewer buds. (These are lab times; colonies occurring naturally in the field grew more rapidly than those raised in the lab, and achieved larger size). Without exception, buds always produced the same (subjective) quantity of spicules as the mother had: all buds of a many-spiculed oozooid developed a heavy coating of spicules, and all buds of a few-spiculed oozooid developed only a sparse coating of spicules. This may explain why in some colonies in the field all the zooids have few spicules, while in other colonies all zooids have many spicules.

An attempt was made to study growth rate in a group of zooids by removing slides from the slide holders every other day for measurement of the zooids settled on them. However, this had an obviously detrimental effect on the zooids: the time to budding was longer, only two buds were produced per oozooid, and nearly all the animals ultimately degenerated, so these data were discarded. Other workers contemplating using this technique should be aware of these possible effects.

Although the process of budding has not previously been described for *Cystodytes,* it is in fact similar to budding in other polycitorids (Oka, 1942; Oka and Usui, 1944; Berrill, 1947, 1948; Levine, 1960; Nakauchi, 1966b, c) and in the polyclinids (Nakauchi, 1966a, 1970, 1974, 1977). *Cystodytes* exhibits Nakauchi's (1966a) Type I budding (abdominal; all buds receive some digestive and epicardial tissue). As in *Amaroucium yamazii* (Nakauchi, 1970), budding in *Cystodytes* is preceded by an elongation and enlargement of the posterior end of the esophagus which begins at least 10 days prior to budding (Fig. 6). This esophageal enlargement will become the new stomach of the mother bud (Figs. 7, 8). A few hours before budding begins, the orange-brown stomach and intestine elongate greatly, as does the epicardium. Feeding ceases, the thorax contracts, and all fecal pellets in the digestive tube collect at the end of the rectum. Budding proceeds posteriorly, with the first constriction occurring at the anterior end of the old stomach. Sometimes the heart of this first bud (called the mother or thoracic bud) can be seen beating at this time. Three or four abdominal buds form, with the terminal bud receiving the mother's old heart. All buds receive some of the orange-brown stomach and intestinal tissue as well as some epicardial tissue. After budding is completed and the mother bud's digestive tube has fully regenerated, the thorax relaxes, the old fecal pellets are ejected, and feeding resumes. Buds begin feeding after 5 to 6 days, and the next budding occurs in about 3½ weeks (lab time). Buds usually orient to the mother but one or two might join a neighboring system; this is probably the reason for the large range in number of zooids per system in Figure 2.

Spicule formation and reallocation at budding

As mentioned earlier, a major difference from the Polyclinidae and from other genera in the Polycitoridae is the formation of calcareous spicules in *Cystodytes.* Using a dissecting microscope at 40 or 80× one can distinguish the first spicules as early as 5 days after settlement. At first, the spicules are very tiny and cannot be individually discerned; only a white spot is noticeable constituting the

entire amount of spicular material. Spicule formation always begins on the inner side of the abdomen at the junction between abdomen and thorax. Gradually the spicules appear to migrate as they increase in number and size until at the end of 4 weeks (laboratory growth time) a zooid's abdomen is completely covered with somewhat overlapping non-birefringent spicules (Fig. 4). These spicules are not attached to the abdomen, though; they are embedded in the innermost layer of tunic that lines the zooid cavity. This layer has been named the "tunic spicular lamina" (TSL). Figure 9 shows the newly forming spicules embedded in the middle of the TSL. Above it are the closely packed bladder cells extending all the way to the outer tunic surface, with the common tunic consisting of the material between the bladder cells. The TSL appears to be completely extracellular, because when a razor blade cross section is made through an adult colony, the zooids can be removed easily from the tunic leaving all the spicules behind. The TSL thus forms a spicular sac in the tunic surrounding but separated from the abdomen of each zooid.

The fate of the spicules at the time of budding was next studied, to determine whether they are dissolved and reformed or simply divided up among the buds in some way. A careful examination at 2 to 3 hr intervals of a few budding zooids showed that first the abdominal epidermal constrictions separated the buds within the zooid cavity. When this process was at least partially completed, the spicular sac began to constrict, resulting ultimately in the abdominal portion of each bud being surrounded by a small spicule sac (Fig. 10). At no time was any dissolution of spicules observed; some of the larger spicules could be followed as they migrated to the buds. During the first stages of spicule "reallocation" many of the spicules were disoriented from their previously regular overlapping pattern. Instead of lying parallel to the abdominal epidermis, they might now be perpendicular to it. This would be expected if the spicules were embedded in a membrane and the entire membrane were constricting. A few spicules were sometimes "left behind" in the test, where they remained isolated. However, active and rapid synthesis of new spicules was also occurring during this time, and approximately 3 to 4 days after the onset of budding all buds were close together but separated from one another by their own spicular sacs, with the spicules regularly aligned parallel to the inner edge of the tunic lining the zooid cavity.

New spicules continued to be added as the buds grew, and the old spicules increased in diameter. Indeed, in adult colonies some spicules may be 1 mm or more in diameter; these might have been carried through a number of generations. They exhibit a complex configuration of knobs not found on small, newly formed spicules (Fig. 11).

Histochemistry of the tunic spicular lamina

Hunt (1970) indicated that in molluscs, the formation of calcium carbonate may depend upon induction by an organic matrix with the presence of sulfated acid mucopolysaccharides being important to this process. Recent papers on tunic composition listed these and other substances found in ascidian tunic and the stains appropriate for their detection (Deck, Hay and Revel, 1966; Smith,

TABLE II.

Histochemistry of C. lobatus *tunic.*

Stain	Specific for	Time (min.)	Tunic region	
			TSL*	Common tunic
0.1% toluidine blue in 30% ethanol	Sulfated acid mucopolysaccharide	10–30	++++ B-metachromatic	++
Aldehyde fuchsin	Sulfated acid mucosubstances	5–15	++	++
Alcian blue pH 2.5	Sulfated acid mucopolysaccharide	14–40	++++	++
Alcian blue pH 1.0	Sulfated acid mucopolysaccharide	15–30	+++	++
Periodic acid-Schiff	Cellulose-like compounds	10	++	++
Sudan black B	Lipid	40	+++	++

*TSL = tunic spicular lamina.

1970; Stiévenart, 1970, 1971). The techniques described by Pearse (1968) for making and using these stains were applied to fresh thin razor blade slices of adult *Cystodytes lobatus* colonies; the results are listed in Table II. Alcian blue and toluidine blue, two stains specific for sulfated acid mucopolysaccharides, stained more heavily around the spicules and inner edge of tunic lining the zooid cavities than elsewhere in the tunic, thus definitely delineating the tunic spicular lamina (TSL). Even those spicules isolated in the tunic during budding and no longer associated with any particular zooids retained this darkly staining membrane around them. (The fact that aldehyde fuchsin did not stain this region in a similar fashion supports Pearse's (1968) statement that it is not the specific stain it has been considered to be).

DISCUSSION

Cystodytes is one of the few genera of ascidians containing mineral concretions that persist in the sediments after the animal's death (Herdman, 1884). In several cases new fossil species of *Cystodytes* have been described solely on the basis of the spicules (Bonet and Benveniste-Velásquez, 1971; Monniot, 1970a; Monniot and Buge, 1971). The taxonomic significance of these spicules depends on the determination of whether or not the spicules form in a species-specific fashion. Monniot (1970b) concluded that polycitorid and didemnid spicules form as a "physico-chemical precipitation of aragonite in the tunic independent of cellular action" and could be used taxonomically only in a general way, to indicate the ascidian group. However, she did intimate that the tunic must play some part in spicule formation due to its fibrous or lamellar structure, but she did not elaborate. The present study proves the existence of an organic matrix for spicule formation (the TSL), differing from the surrounding common tunic in the concentration of sulfated acid mucopolysacharides. Also, the newly forming spicules have a lumen and are incompletely mineralized (Fig. 9c), while the spicules of adult *C. lobatus* are completely mineralized; this may indicate

another organic matrix for mineralization within each spicule (Lowenstam, personal communication). Cellular action is implicated by the fact that the spicules form in a particular region of the animal, at the junction between abdomen and thorax, and migrate out from that point to the TSL. In addition, a genetic component is implied by the great variation among colonies in the extent of spicule formation. Lafargue and Kniprath's (1978) paper proves the cellular origin of spicules in the Didemnidae. They identified the organ of spicular origin and also found that the spicules are surrounded by a discrete double-layered membrane. Thus their study and this study contradict the findings of Prenant (1925), Pérès (1948) and Monniot (1970b). Pérès was obviously troubled by his inability to determine the reasons for tunic stratification in the Polyclinidae, which he stated was especially noticeable around the periphery of zooids in addition to the thin outer cuticle.

Examination of the spicules in *Cystodytes lobatus* was difficult because nearly any treatment (fixation, sectioning, staining, even relaxation of live zooids) usually resulted in some disruption of the bladder cells and partial dissolution of the spicules. This is why all of the staining was done supravitally, as suggested by Pearse (1968). Nevertheless, some stains (aldehyde fuchsin and alcian blue pH 1.0 especially) changed the spicules into needle-like clusters within a few minutes. Fixation in alcohol or buffered formalin also disrupted bladder cell membranes, and after being left for a few weeks in fixative the surface irregularities disappeared from many spicules in adult colonies. Indeed, some *Cystodytes* colonies after several years on a museum shelf have few if any recognizable spicules left, a fact which undoubtedly has led to erroneous calculations of the abundance and type of spicules when the colonies were alive (Ritter, 1900; Van Name, 1945; Millar, 1962). Because of these difficulties, taxonomic descriptions of *Cystodytes* and didemnid species should include photographs or at least descriptions of spicules from live colonies if possible.

The question remains of how the tunic spicular lamina can "bud" when the zooid buds, a problem especially puzzling since the lamina appears to be completely extracellular. At a magnification of 675×, it is possible that a membrane was indistinctly seen in the 1-μm sections between the outer edge of the tunic spicular lamina and the beginning of the bladder cell region. If this is so, perhaps this membrane somehow separates from the rest of the tunic during budding. It will be necessary to embed and section zooids in the process of budding in order to examine this further.

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gestions. My husband Charles spent much time in helping me with all aspects of the photography, microscopy, pH determinations and reading of the manuscript; I greatly appreciate his constant support throughout this study.

Summary

1. The colonial ascidian *Cystodytes lobatus* has a long breeding season (at least 6 months) and releases tadpoles sporadically throughout the day, indicating a long period of recruitment.

2. Tadpoles of *C. lobatus* were settled and reared in the laboratory in order to observe early growth, budding and spicule formation.

3. Budding is preceded by the formation of a new stomach at the posterior end of the esophagus and fits Nakauchi's Type I budding pattern.

4. Spicule formation begins within 5 days after settlement. The spicules appear to form in a particular region at the anterior end of the abdomen and migrate over the abdomen to form a single or slightly overlapping layer embedded in a "tunic spicular lamina." This lamina lies between the common tunic and the zooid cavity and forms a spicular sac in the tunic surrounding but separated from the abdomen of each zooid. It stains especially heavily for sulfated acid mucopolysaccharide; the spicules are concluded to form by cellular action in this organic matrix.

5. There is great variation among zooids in the quantity of spicules formed. These differences are maintained in the buds, resulting in colonies in which all zooids either have few or many spicules, and are therefore probably genetic in origin.

6. During budding the spicular sac becomes disrupted and appears to bud, resulting in a reallocation of the spicules to the buds and formation of separate spicular sacs around the abdomen of each bud. At budding there is apparently no disruption of bladder cell membranes in the tunic and no dissolution of spicules by the acids contained in the bladder cells.

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